

Osteoprotegerin Is a New Regulator of Inflammation and Angiogenesis in Proliferative Diabetic Retinopathy

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PURPOSE. Osteoprotegerin (OPG) is a novel regulator of endothelial cell function, angiogenesis, and vasculogenesis. We correlated expression levels of OPG with those of the angiogenic and inflammatory factors vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1/CCL2) in proliferative diabetic retinopathy (PDR). We also examined expression of OPG in retinas from diabetic rats and diabetic patients and measured production of OPG by human retinal microvascular endothelial cells (HRMEC) and investigated its angiogenic activity.

METHODS. Vitreous samples from 47 PDR and 28 nondiabetic patients, epiretinal membranes from 14 patients with PDR, human retinas (10 from diabetic patients and 10 from nondiabetic subjects), and rat retinas and HRMEC were studied by using enzyme-linked immunosorbent assay, immunohistochemistry, immunofluorescence, Western blot analysis, and RT-PCR. In vitro and in vivo angiogenesis assays were performed.

RESULTS. We showed a significant increase in the expression of OPG, VEGF, and MCP-1/CCL2 in a comparison between vitreous samples from PDR patients and those from nondiabetic controls. Significant positive correlations were found between levels of OPG and levels of VEGF and MCP-1/CCL2. In epiretinal membranes, OPG was expressed in vascular endothelial cells and stromal cells. Significant increases of OPG mRNA and protein were detected in the retinas from diabetic patients. The proinflammatory cytokines TNF- α and IL-1 β , but not VEGF, MCP-1/CCL2 or thrombin, induced upregulation of OPG in HRMEC. Osteoprotegerin induced ERK1/2 and Akt phosphorylation in HRMEC and stimulated their migration. Osteoprotegerin potentiated the angiogenic effect of VEGF in the in vivo protein gelatin plug assay.

CONCLUSIONS. These results suggest that OPG is involved in PDR angiogenesis.

Keywords: angiogenesis, monocyte chemoattractant protein-1, osteoprotegerin, proliferative diabetic retinopathy, vascular endothelial growth factor

Ischemia-induced retinal angiogenesis and vasculogenesis are hallmark features of proliferative diabetic retinopathy (PDR) and are critical steps for the development and progression of PDR.^{1–4} Pathologic growth of new blood vessels and expansion of extracellular matrix in association with the outgrowth of fibrovascular epiretinal membranes at the vitreoretinal interface often leads to catastrophic loss of vision due to vitreous hemorrhage and/or traction retinal detachment. The dynamic balance between proangiogenic and antiangiogenic factors is thought to regulate angiogenesis.⁵ Vascular endothelial growth factor (VEGF) is the major angiogenic factor in PDR that promotes neovascularization and vascular leakage.⁶ In several studies, the overexpression of proinflammatory and proangiogenic molecules was demonstrated in the ocular microenvironment of patients with PDR,^{7–12} suggesting that persistent

inflammation and neovascularization are critical for PDR initiation and progression.

Our understanding of the precise molecular mechanisms responsible for the regulation of retinal angiogenesis and vasculogenesis in patients with PDR is incomplete and molecular regulation of these processes is the subject of many studies. A potential novel regulator of endothelial cell function, angiogenesis, and vasculogenesis is osteoprotegerin (OPG), a member of the TNF receptor superfamily. Osteoprotegerin lacks a transmembrane domain and, unlike most members of this family, is secreted into the extracellular space.^{13,14} Osteoprotegerin was initially identified for its role in regulating bone metabolism by mediating paracrine signaling between osteoblasts and osteoclasts.^{13–16} In addition to its role in bone metabolism, OPG has recently been found to have additional



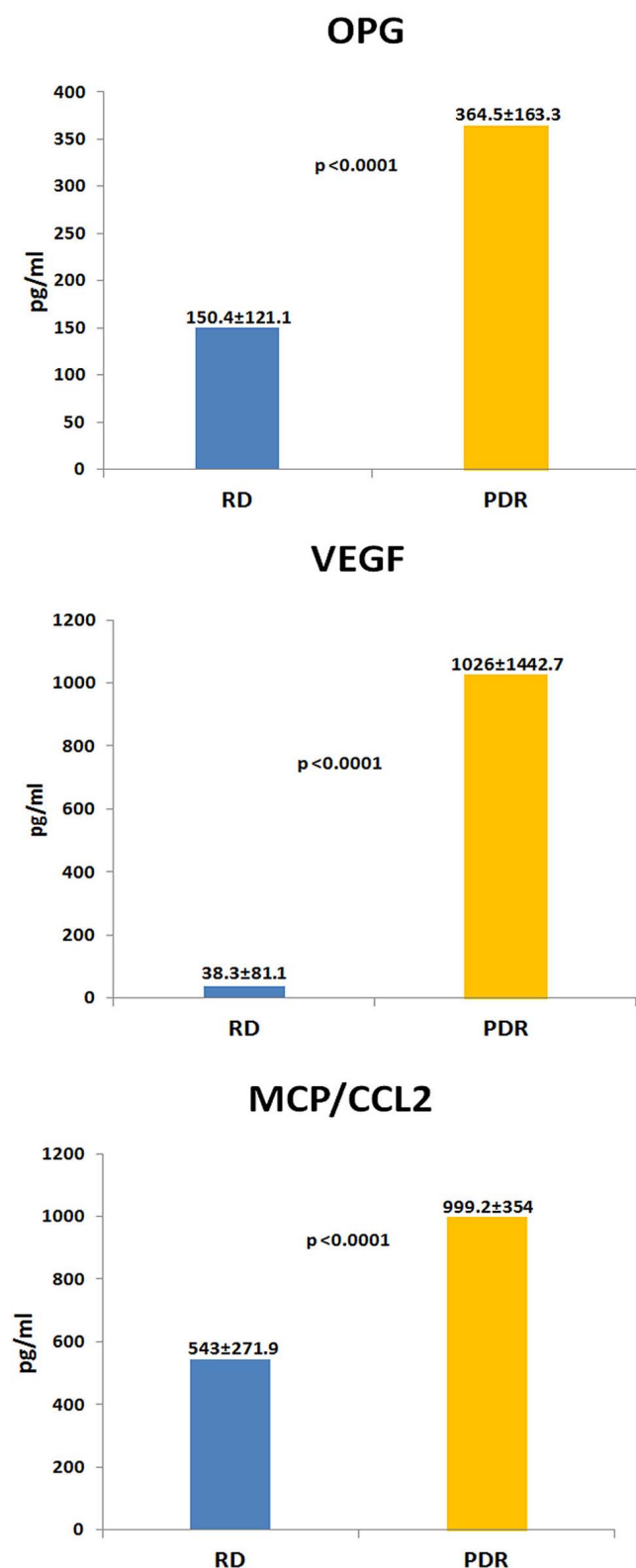


FIGURE 1. Comparisons of mean OPG, VEGF, and MCP-1/CCL2 ELISA levels in vitreous fluid samples from patients with PDR ($n = 47$) and those from nondiabetic patients with rhegmatogenous RD ($n = 28$). The difference between the two means was statistically significant at 5% level of significance.

roles in regulating endothelial cell function in tumor angiogenesis and vascular disease.^{13,14} In addition, OPG may be involved in development of vascular endothelial cell dysfunction and micro- and macrovascular complications in diabetes.^{17,18} The expression of OPG in PDR has not been reported so far. Given the key roles of OPG in regulating endothelial cell function and promotion of angiogenesis, we investigated the hypothesis that OPG may be involved in PDR pathogenesis.

MATERIALS AND METHODS

Vitreous Fluid Samples and Epiretinal Membranes Specimens

Undiluted vitreous fluid samples were obtained from 47 PDR patients during pars plana vitrectomy as described previously.⁷⁻¹² The indications for vitrectomy were tractional retinal detachment and/or nonclearing vitreous hemorrhage. The control group consisted of 28 patients who had undergone vitrectomy for the treatment of rhegmatogenous retinal detachment with no proliferative vitreoretinopathy (PVR). Controls were free from systemic disease. Epiretinal fibrovascular membranes were obtained from 14 PDR patients during pars plana vitrectomy for the repair of tractional retinal detachment. Control epiretinal membranes were obtained from 10 nondiabetic patients undergoing vitreoretinal surgery for retinal detachment complicated by PVR. Membranes were fixed for 2 hours in 10% formalin solution and embedded in paraffin.

The study was conducted according to the tenets of the Declaration of Helsinki. All patients were candidates for vitrectomy as a surgical procedure. All patients signed a preoperative informed written consent and approved the use of the excised epiretinal membranes and vitreous fluid for clinical research. The study design and protocol were approved by the Research Centre and Institutional Review Board of the College of Medicine, King Saud University.

Human Postmortem Eyes

Human postmortem eyes were obtained from 10 diabetic individuals with mild nonproliferative diabetic retinopathy and 10 nondiabetic (control group) donors matched by age. After enucleation, one eye from each donor was snap-frozen in liquid nitrogen at -80°C and stored until assayed for mRNA or protein analyses. Neuroretina and retinal pigment epithelium (RPE) were quickly harvested. Vitreous and neuroretinas were removed, and the RPE layer was carefully peeled from Bruch's membrane, using forceps (Dumont no. 5; Sigma, Madrid, Spain) under a dissecting microscope (model SZ61; Olympus, Barcelona, Spain). The other eye was fixed in 4% paraformaldehyde and embedded in paraffin.

All ocular tissues were used in accordance with applicable laws and with tenets of the Declaration of Helsinki for research involving human tissue. In addition, this study was approved by the Ethics Committee, Institut de Recerca Hospital Universitari Vall d'Hebron.

Rat Streptozotocin-Induced Diabetes Model

All procedures with animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the College of Pharmacy, King Saud University. Diabetes was induced in adult male Sprague-Dawley rats, by intraperitoneal injection of strepto-

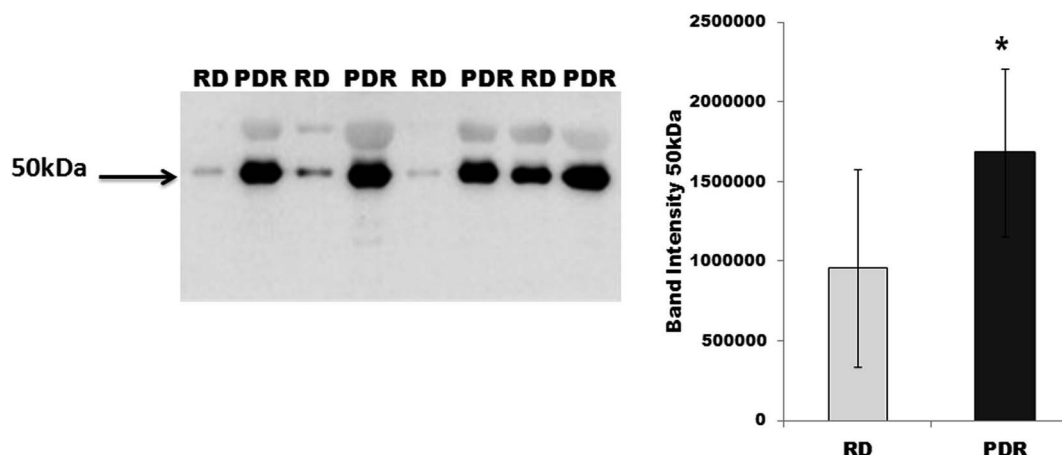


FIGURE 2. Determination of OPG levels in vitreous fluid. Equal volumes (15 μ L) of vitreous fluid samples from patients with PDR ($n = 16$) and from nondiabetic patients with rhegmatogenous RD ($n = 8$) were analyzed using PAGE, and the presence of OPG was detected by using Western blot analysis. A representative set of samples (three RD samples and three PDR samples) is shown (*left panel*). The intensity of the protein band around 50 kDa was determined in all samples (*right panel*). * $P < 0.05$ (Mann-Whitney U test).

zotocin (Sigma) as described previously.⁷ After 4 and 12 weeks of diabetes, animals were sacrificed, and retinas were dissected, flash frozen, and stored at -70°C . Similarly, retinas were obtained from age-matched nondiabetic control rats.

Enzyme-Linked Immunosorbent Assays for Vitreous Samples

Enzyme-linked immunosorbent assay (ELISA) kits for human osteoprotegerin (catalog no. DY-805), MCP-1/CCL2 (catalog no. DCP00), and VEGF (catalog no. SVE00) were purchased from R&D Systems, Minneapolis, MN, USA.

Western Blot Analysis for OPG Expression in Human Vitreous Fluid Samples and Rat Retinas

Retinas from diabetic and control rats were homogenized in Western blot lysis buffer as described previously,⁷ and homogenates were subjected to sodium dodecyl sulfate (SDS)-PAGE. Equal volumes (15 μ L) of vitreous samples were boiled (10 min) in Laemmli's sample buffer (1:1 v/v) under reducing conditions and analyzed as described previously.^{7,11,12} Blots were incubated overnight with anti-OPG antibody (1:1000 dilution; clone ab73400; Abcam, Cambridge, UK) at 4°C .

Immunohistochemical Staining of Epiretinal Membranes

For CD31, α -smooth muscle actin (α -SMA), and OPG detection, antigen retrieval (10-minute incubation) was performed using citrate based buffer (pH 5.9 to 6.1; Bond epitope retrieval solution 1; Leica Biosystems, Buffalo Grove, IL, USA). For CD45 detection, antigen retrieval (20-minute incubation) was performed using Tris/EDTA buffer (pH 9) (Bond epitope retrieval solution 2; Leica). Subsequently, the sections were incubated (60 minutes) with mouse monoclonal anti-CD31 antibody (clone JC70A; Dako, Glostrup, Denmark), mouse monoclonal anti-CD45 antibody (clones 2B11 and PD7/26; Dako), mouse monoclonal anti- α -SMA antibody (clone 1A4; Dako), and rabbit polyclonal anti-OPG antibody (1:50 dilution; clone ab9986; Abcam) as described.^{3,7,11,12} To identify the phenotype of cells expressing OPG, sequential double immunohistochemistry was performed as described.^{3,7,11,12}

Immunoreactive blood vessels and cells were counted in five representative fields using an eyepiece calibrated grid in combination with the $40\times$ objective. With this magnification and calibration, immunoreactive blood vessels and cells present in an area of 0.33×0.22 mm were counted.

Immunofluorescent Detection of OPG in Retinas From Diabetic Patients

Retinal sections (7- μ m thickness) were pretreated as described,¹⁹ blocked (2% bovine serum albumin in 0.05% Tween in phosphate-buffered saline for 1 hour), and incubated overnight with primary antibody against OPG (1:500 dilution; clone ab73400; Abcam). Colocalization studies were performed using anti-glial fibrillary acidic protein (GFAP) (1:1000 dilution; clone ab10062; Abcam), anti-Iba1 (1:200 dilution; clone ab5076; Abcam), and anti-collagen IV (1:50 dilution; clone ab6311; Abcam). After sections were washed, they were incubated with Alexa Fluor 488 or 594 secondary antibodies (Molecular Probes, Invitrogen, Madrid, Spain) at room temperature for 1 hour. Slides were cover-slipped with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei (Vector Laboratories, Pales, Sant Cugat del Vallés, Spain). Images were acquired with a confocal laser scanning microscope (model FV1000; Olympus).

Analysis of OPG Expression in Retinas From Diabetic Patients

Human neuroretina and RPE were harvested under the microscope. For Western blot analysis, neuroretina and RPE samples were extracted with radioimmunoprecipitation assay buffer and homogenized by sonication. The protein concentration was determined using a bicinchoninic acid protein assay (Bio-Rad Laboratories, Madrid, Spain). Protein extracts (20 μ g) were resolved by 10% SDS-PAGE and transferred to ECL nitrocellulose membranes (Hybond; Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were first incubated with anti-OPG (1:1000 dilution; clone ab73400; Abcam) and afterward with peroxidase-conjugated secondary antibody (Bio-Rad Laboratories). Proteins were visualized using the enhanced chemiluminescence detection system (Supersignal CL-horseradish peroxidase substrate system; Thermo Scientific, Rockford, IL, USA).

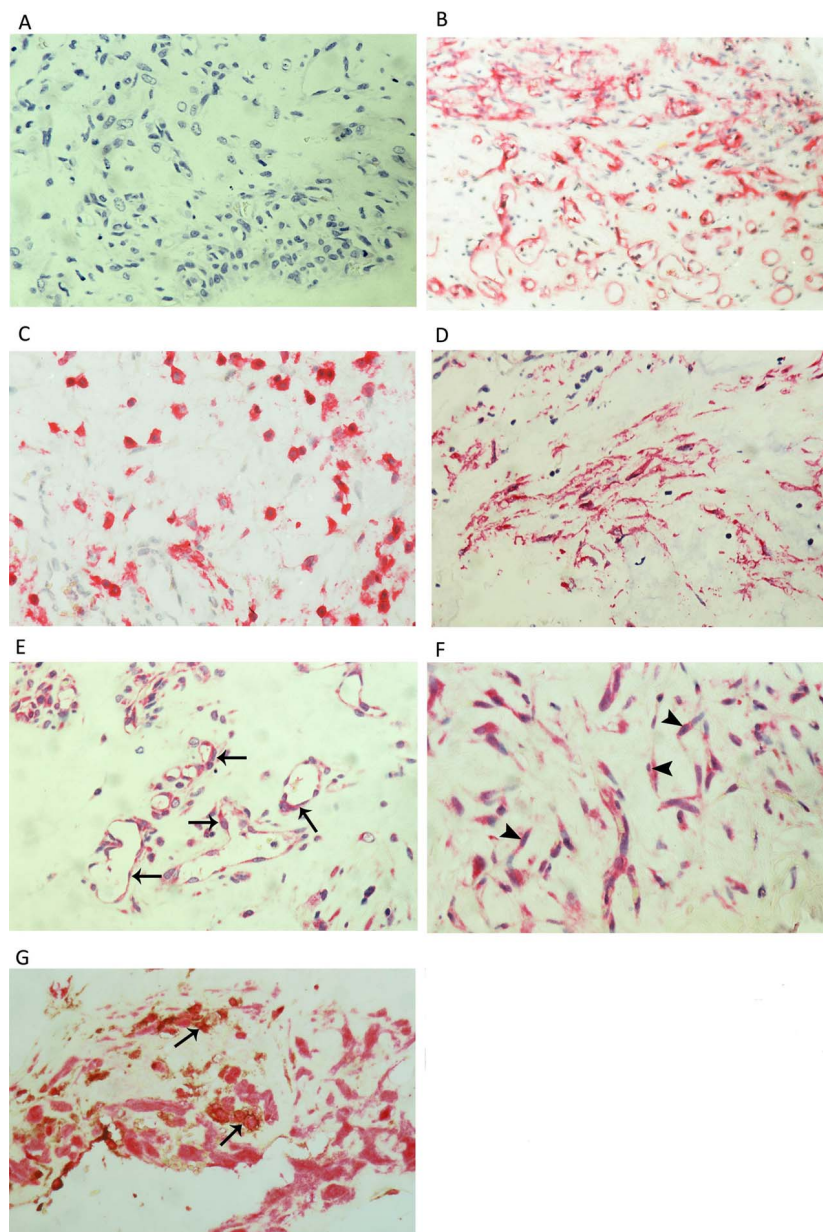


FIGURE 3. Osteoprotegerin is expressed in epiretinal membranes from patients with PDR. Expression of OPG in PDR epiretinal membranes was investigated by immunohistochemistry analysis using a specific anti-OPG antibody. Specificity was checked by use of an irrelevant polyclonal antibody (A) (negative control). Microvessels, infiltrating leukocytes, and myofibroblasts were stained using anti-CD31 (B), anti-CD45 (C), and anti- α -smooth muscle actin (D) antibodies, respectively. Immunoreactivity for OPG was observed in the vascular endothelium (arrows) (E) and in stromal spindle-shaped cells (arrowheads) (F). Double-immunohistochemistry analysis was performed using CD45 (brown) and OPG (red), and co-staining was detected in stromal cells (arrows) (G) (original magnification $\times 40$).

Total RNA was extracted from isolated retinal tissues by using Trizol reagent (Invitrogen). Concentrations of RNA were determined, and reverse transcription was performed using 1 μ g of total RNA, using random hexanucleotide priming and reagents provided by Applied Biosystems, Madrid, Spain.

The power SYBR Green PCR Master Mix (Applied Biosystems) was used for real-time PCR, and reactions were conducted at 95°C for 10 min, for 50 cycles of 15 seconds each at 95°C, and for 1 minute at 60°C, using the Prism 7000 unit (Applied Biosystems). Each sample was assayed in duplicate, and control negative samples were included in each experiment. Specific primer pairs corresponding to human OPG (forward primer 5'-TGGCACCAAAGTAAACGCAGAG-3' and reverse primer 5'-CTCGAAGGTGAGGTTAGCATGTC-3') and

human β -actin (as endogenous control: forward primer 5'-TGGAGAAAATCTGGCACCAC-3' and reverse primer 5'-GAGGCGTACAGGGATAGCAC-3') were used.

Induction of Human Retinal Microvascular Endothelial Cells

Human retinal microvascular endothelial cells (HRMEC; Cell Systems, Kirkland, WA, USA) were cultured as described.¹⁰ Confluent monolayers were stimulated with VEGF (Biolegend, San Diego, CA, USA), IL-1 β (Peprotech, Rocky Hill, NJ, USA), TNF- α (Peprotech), MCP-1/CCL2 (Peprotech) and thrombin (Sigma). In addition, 25 mM of mannose (osmotic control) or 25 mM of glucose was added to the stimulation medium to evaluate

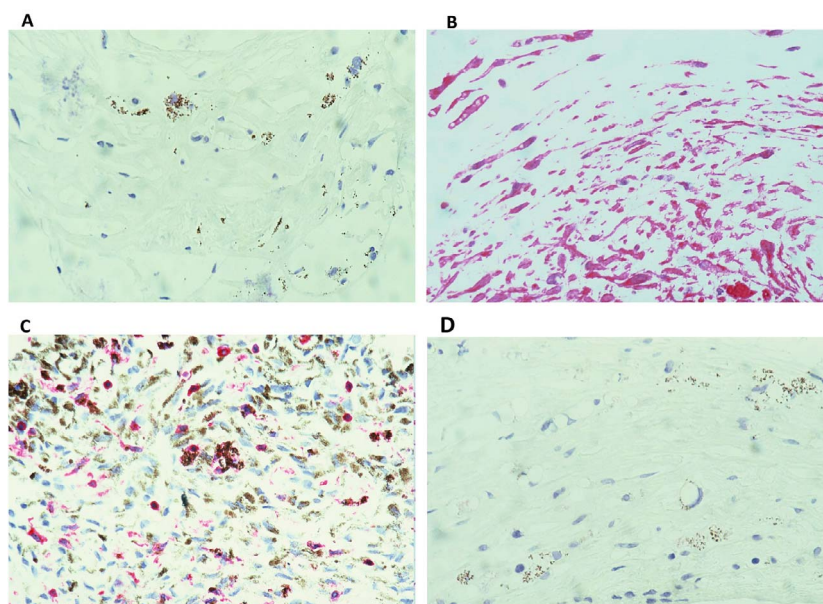


FIGURE 4. Immunostaining of proliferative vitreoretinopathy fibrocellular epiretinal membranes. Immunohistochemical staining for CD31 showing no immunoreactivity (A). Immunohistochemical staining for α -smooth muscle actin shows immunoreactivity in spindle-shaped myofibroblasts (B). Immunohistochemical staining for CD45 shows immunoreactivity in leukocytes (C). Immunohistochemical staining for osteoprotegerin shows no immunoreactivity (D) (original magnification $\times 40$).

the effect of hyperglycemia. All inductions were performed in duplicate. After 72 hours, cell supernatants were harvested, centrifuged to remove cell debris and stored at -20°C .

Signal Transduction Assays for Phosphorylated Akt and Extracellular Signal-Regulated Kinase (ERK)1/2

Signal transduction experiments with HRMEC were performed as described previously.¹⁰

Chemotaxis Assay

The chemotactic activity of recombinant human OPG (TNFRSF11B; R&D Systems) for HRMEC was evaluated using cell invasion/migration plates and the RTCA-DPx CELLigence instrument (Acea Biosciences, San Diego, CA, USA). This system records changes of electrical impedance, which is expressed as cell index (CI) over a prolonged time course. First, 160 μL of stimulus (control medium, OPG, or VEGF) and 50 μL of EBM-2 with 0.4% fetal bovine serum (FBS; control medium) was added to the lower and upper chambers, respectively. Subsequently, the CIM16-plate was equilibrated (1 hour, 37°C). Afterward, HRMECs (40,000 cells per well; 100 μL /well) were seeded in the upper chamber in EBM-2 medium containing 0.4% FBS and allowed to settle onto the membrane (30 minutes, 20°C). After transfer to a CO_2 incubator (37°C), the impedance value was monitored every minute during 15 hours and expressed as CI. Cells migrating across the membrane cause an increase in the CI. Each experiment was performed in duplicate.

In Vivo Gelatin Protein Plug Assay

Cooled growth factor-reduced protein gelatin (9 to 11 mg of protein/mL; Matrigel; BD Biosciences, San Jose, CA, USA) was injected subcutaneously (500 μL /mouse) into the flank of 7-8 week-old female C57/Bl6 mice. The first group of mice were injected pure protein gelatin as control, others received protein gelatin mixed with either VEGF (250 ng), OPG (500

ng), or VEGF (250 ng) plus OPG (500 ng). After 7 or 8 days, plugs were resected and digested for 1 hour at 37°C with dispase (15 U/mL) in the presence of DNase (10,000 U/mL) to obtain single-cell suspensions. The total number of cells recovered from each plug was determined, and the cell suspensions were incubated with antibodies against CD45 and CD34 (clones 30-F11 and RAM34; eBioScience, San Diego, CA, USA), to distinguish between leukocytes and endothelial cells, respectively. Using flow cytometry the number of endothelial cells recruited into the plugs was determined.

Statistical Analysis

Data are mean \pm SD or SEM. The nonparametric Mann-Whitney *U* test was used to compare means from two independent groups. Pearson correlation coefficients were computed to investigate correlation between variables. A *P* value less than 0.05 indicated statistical significance. SPSS version 20.0 software (IBM, Armonk, NY, USA) for Windows (Microsoft, Redmond, WA, USA) was used for statistical analysis.

RESULTS

ELISA Levels of OPG, VEGF, and MCP-1/CCL2 in Vitreous Samples From PDR Patients

OPG was detected in 24 of 28 vitreous samples (86%) from nondiabetic control patients, as well as in 45 of 47 PDR samples (96%). Mean OPG level in PDR patients (364.5 ± 163.3 pg/mL) was significantly higher than mean level in nondiabetic control patients (150.4 ± 121.1 pg/mL) ($P < 0.0001$). VEGF was detected in 17 of 28 samples (60.7%) from controls, and in 38 of 47 PDR samples (80.9%). Mean VEGF level in PDR patients (1026 ± 1442.7 pg/mL) was significantly higher than mean level in controls (38.3 ± 81.1 pg/mL; $P < 0.0001$). MCP-1/CCL2 was detected in 25 of 28 samples (89%) from controls and in 45 of 47 samples (96%) samples from PDR patients. Mean MCP-1/CCL2 level in PDR patients (999.2 ± 354 pg/mL) was

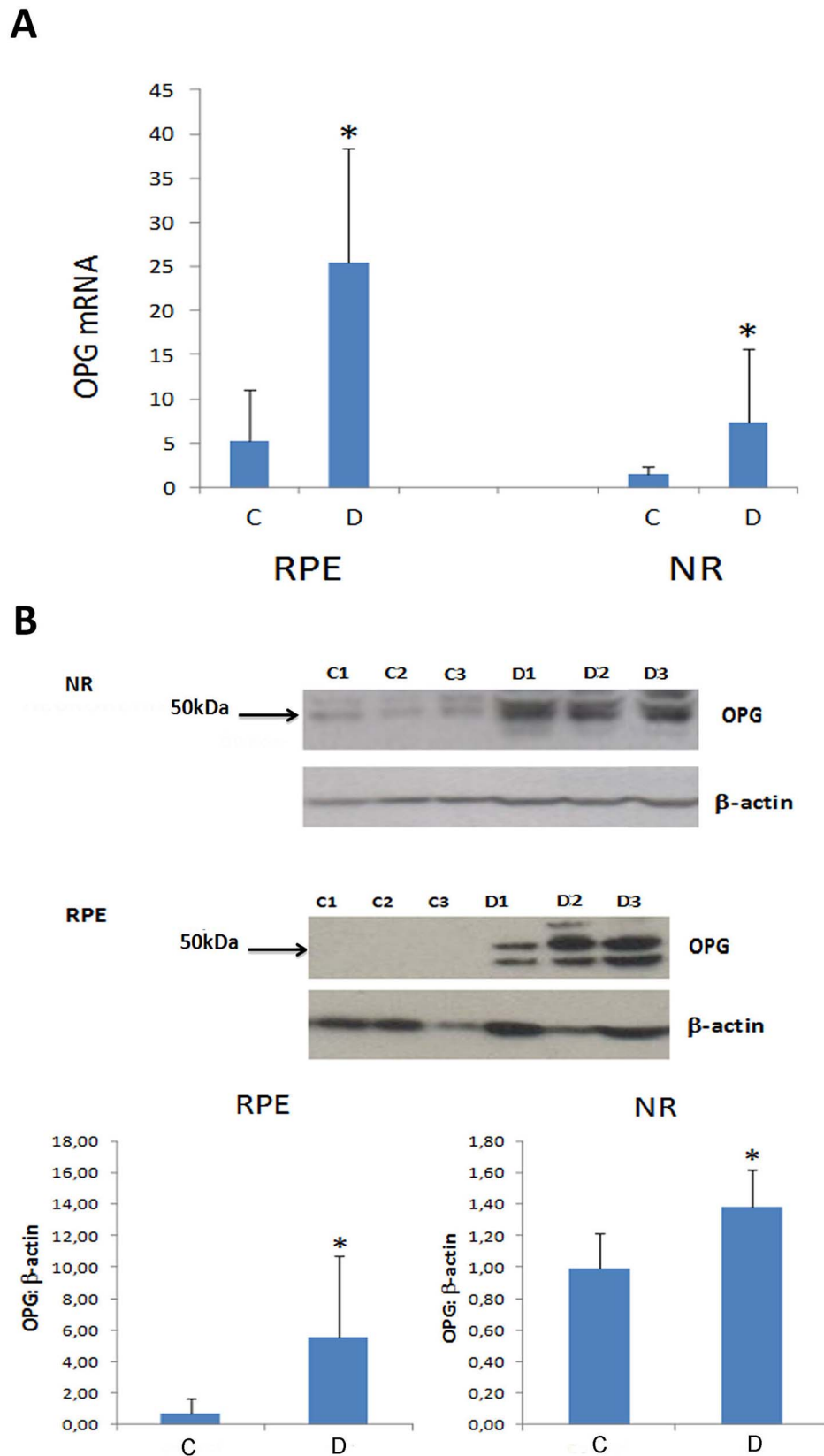


FIGURE 5. (A) Osteoprotegerin mRNA is more abundant in retinas from subjects with diabetes. Expression of OPG mRNA was analyzed by using real-time quantitative RT-PCR in retinal RPE and neuroretina (NR). Osteoprotegerin mRNA expression was calculated after normalization of β -actin mRNA levels. Bars represent mean \pm SD of the relative quantification (RQ) values obtained for mRNA levels in diabetic (D) ($n = 10$) and nondiabetic (C) ($n = 10$) donors. * $P < 0.05$ (Mann-Whitney U test). (B) Osteoprotegerin protein expression is upregulated in retinas from subjects with diabetes. Osteoprotegerin protein expression was determined by Western blot analysis on lysates of RPE and NR from representative diabetic (D) ($n = 3$) and nondiabetic (C) ($n = 3$) donors. Intensities of the protein bands around 50 kDa were determined in all samples ($n = 10$ /group). Data (arbitrary units) are mean \pm SD. * $P < 0.05$ (Mann-Whitney U test).

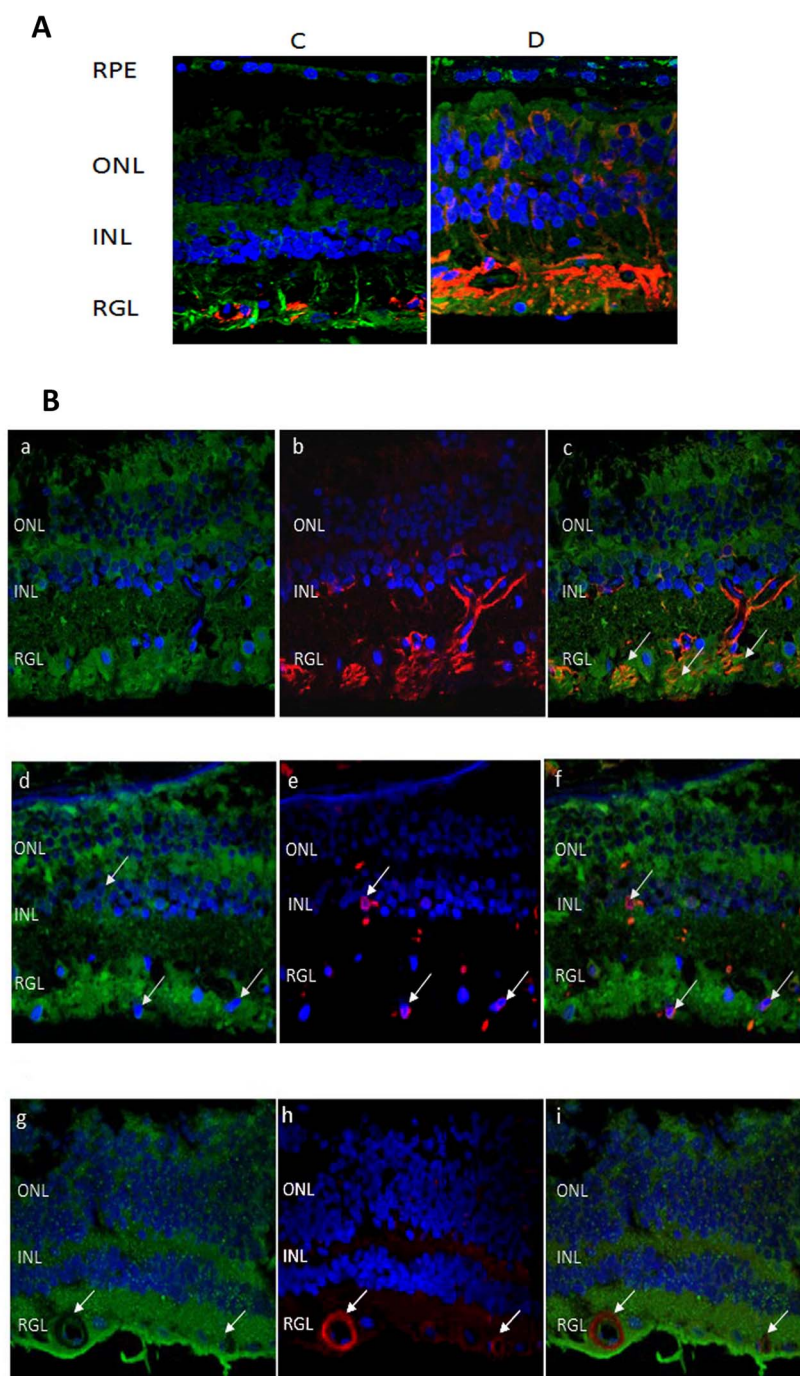


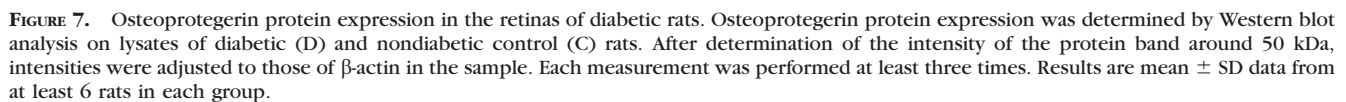
FIGURE 6. (A) Microscopy analysis of OPG expression in human retina. Immunofluorescent staining of OPG in representative samples from nondiabetic (C) and diabetic (D) donors. After fixation, retinas were stained with a polyclonal rabbit anti-OPG antibody (green) and anti-glial fibrillary acidic protein antibody (red) to show glial activation. Nuclei were labeled with DAPI (blue). INL, inner nuclear layer; ONL, outer nuclear layer; RGL, retinal ganglion layer. (B) Osteoprotegerin colocalized with GFAP, Iba1, and collagen IV in human diabetic retina. (a) Osteoprotegerin immunofluorescence (green). (b) GFAP immunofluorescence (red). (c) Osteoprotegerin (green); GFAP (red), and nuclei (DAPI stain) (blue). Orange fluorescence (white arrows) shows colocalization of both fluorescent colors. (d) Osteoprotegerin immunofluorescence (green). (e) Iba1 immunofluorescence (red). (f) Osteoprotegerin (green), Iba1 (red), and nuclei (blue). Orange fluorescence (white arrows) shows colocalization of both fluorescent colors. (g) Osteoprotegerin (green). (h) collagen IV (red). (i) Osteoprotegerin (green), collagen IV (red), and nuclei (blue). Orange fluorescence (white arrows) shows colocalization in retinal vessels. INL, inner nuclear layer; ONL, outer nuclear layer; RGL, retinal ganglion layer.

significantly higher than mean level in nondiabetic control patients (543 ± 271.9 pg/mL; $P < 0.0001$) (Fig. 1).

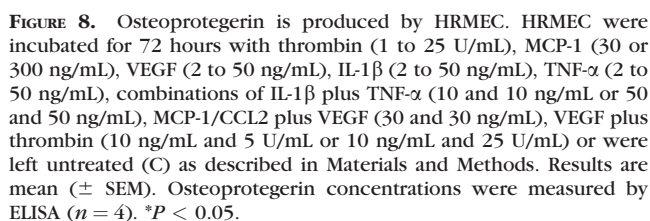
Significant positive correlations were found between vitreous fluid levels of OPG and levels of VEGF ($r = 0.33$; $P = 0.006$) and MCP-1/CCL2 ($r = 0.41$; $P = 0.001$). In contrast, the vitreous fluid levels of VEGF and MCP-1/CCL2 did not correlate.

Western Blot Analysis of Vitreous Samples

Using Western blot analysis, we confirmed that OPG was present in vitreous samples and that OPG expression was enhanced in PDR. Osteoprotegerin migrated at the position of its expected molecular weight and indeed, densitometric



To identify the cellular source of vitreous fluid OPG, fibrovascular epiretinal membranes from patients with PDR were studied using immunohistochemical analysis. No staining



The number of blood vessels that were immunoreactive for OPG ranged from 4 to 60, with a mean of 15.9 ± 17.3 per specimen. The number of OPG-positive stromal cells ranged from 3 to 105 cells, with a mean of 34.4 ± 36.9 per specimen. Significant positive correlations were detected between the numbers of blood vessels expressing CD31 and the numbers of blood vessels ($r=0.89$; $P<0.0001$) and stromal cells ($r=0.56$; $P=0.036$) expressing OPG.

OPG mRNA expression was higher in diabetic donors than in nondiabetic donors in both RPE and neuroretina ($P < 0.001$ for both comparisons) (Fig. 5A). Western blot analysis demonstrated that OPG protein expression was upregulated in diabetic retinas compared to nondiabetic control retinas in RPE as well as in neuroretina ($P < 0.001$ for both comparisons) (Fig. 5B). Immunostaining for OPG was observed in the cytoplasm of all

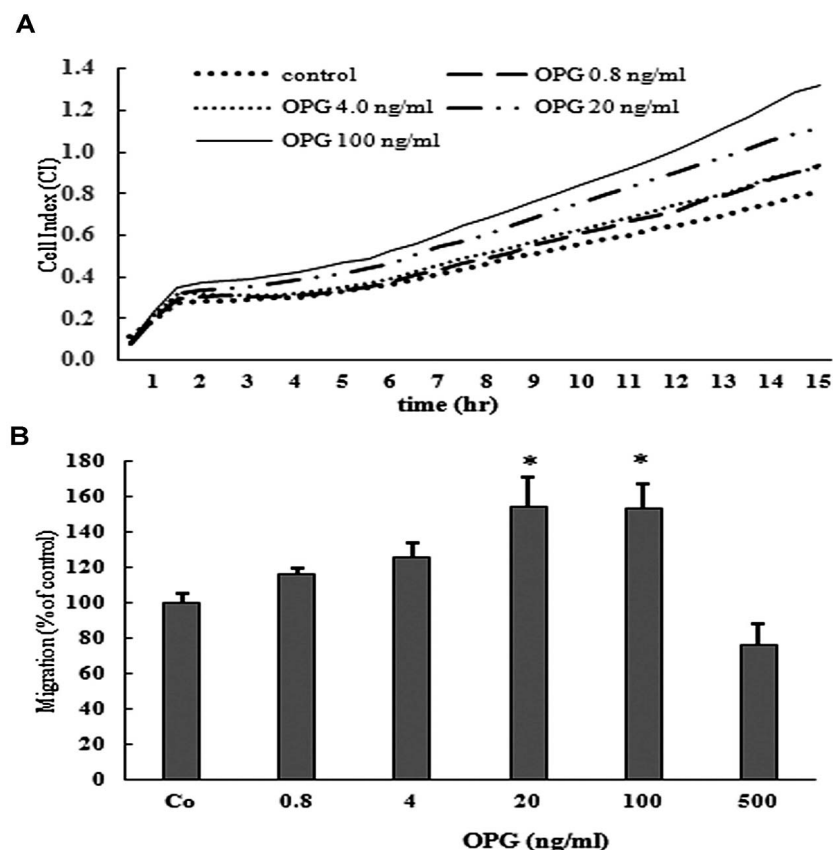


FIGURE 9. Osteoprotegerin stimulates retinal endothelial cell migration. HRMEC were treated with recombinant human OPG at the indicated concentrations. Migration was monitored for 15 hours, using the xCELLigence system. (A) Representative experiment. (B) Cell indexes recorded after 15 hours of incubation were divided by the average index from cells migrating toward dilution buffer (C). Results (mean percentage \pm SEM) are derived from 3 or 4 independent experiments. * $P < 0.05$.

retinal layers (Fig. 6A). In addition, double immunofluorescence analysis revealed that OPG was colocalized with the glial cell marker GFAP, the microglial cell marker Iba1 and the basal lamina marker collagen IV (Fig. 6B).

We quantified the expression of OPG in rat retinas by Western blot analysis. Densitometric analysis of the bands revealed that the expression of OPG did not differ significantly between nondiabetic controls and diabetic rat retinas after 4 and 12 weeks of acutely induced diabetes (Fig. 7).

Human Retinal Microvascular Endothelial Cells Produce OPG In Vitro

To confirm our findings that vascular endothelial cells in PDR fibrovascular epiretinal membranes express OPG, we investigated whether HRMEC could produce OPG. To reach that goal, we treated HRMEC for 72 hours with cytokines or growth factors known to be present in the ocular microenvironment of patients with PDR, in particular MCP-1/CCL2, VEGF, thrombin, IL-1 β , and TNF- α . Unstimulated HRMEC did release a significant amount of OPG (about 45 pg/mL) in the culture medium (Fig. 8). The production of OPG by HRMEC was not upregulated in response to MCP-1/CCL2, VEGF or thrombin added as single stimulus or in combination. However, the cytokines IL-1 β and TNF- α alone or in combination did increase the levels of OPG in the culture medium. In response to IL-1 β plus TNF- α up to 15 ng/mL of OPG was detected by specific ELISA. Raising the glucose concentration of the culture medium to 25 mM did not

affect spontaneous and stimulated release of OPG (data not shown).

OPG Induces Human Retinal Microvascular Endothelial Cell Migration

In order to investigate whether OPG affected HRMEC migration, real-time impedance-based recording of migration was performed using the xCELLigence system. HRMEC were stimulated with different doses of OPG, and migration was followed over 15 hours. Figure 9A shows the recorded cell indexes from one representative experiment. In total 4 experiments were performed and statistically evaluated. When results were expressed relative to the cell index of spontaneously migrating HRMEC, OPG significantly stimulated endothelial cell migration at 20 and 100 ng/mL ($154 \pm 17\%$ and $153 \pm 14\%$, respectively) (Fig. 9B). For comparison, VEGF, a potent inducer of endothelial cell migration, was also used as chemoattractant. VEGF at 5 ng/mL significantly increased endothelial cell migration to $337 \pm 42\%$ (data not shown).

OPG Activates Signal Transduction Pathways in Human Retinal Microvascular Endothelial Cells

Next, we verified whether OPG was able to activate ERK1/2 and Akt signal transduction pathways. Similar to the angiogenic factor VEGF (30 ng/mL), OPG induced significant phosphorylation of these two kinases (Fig. 10). The minimal effective concentration of OPG, 300 ng/mL was, however, at least 10-

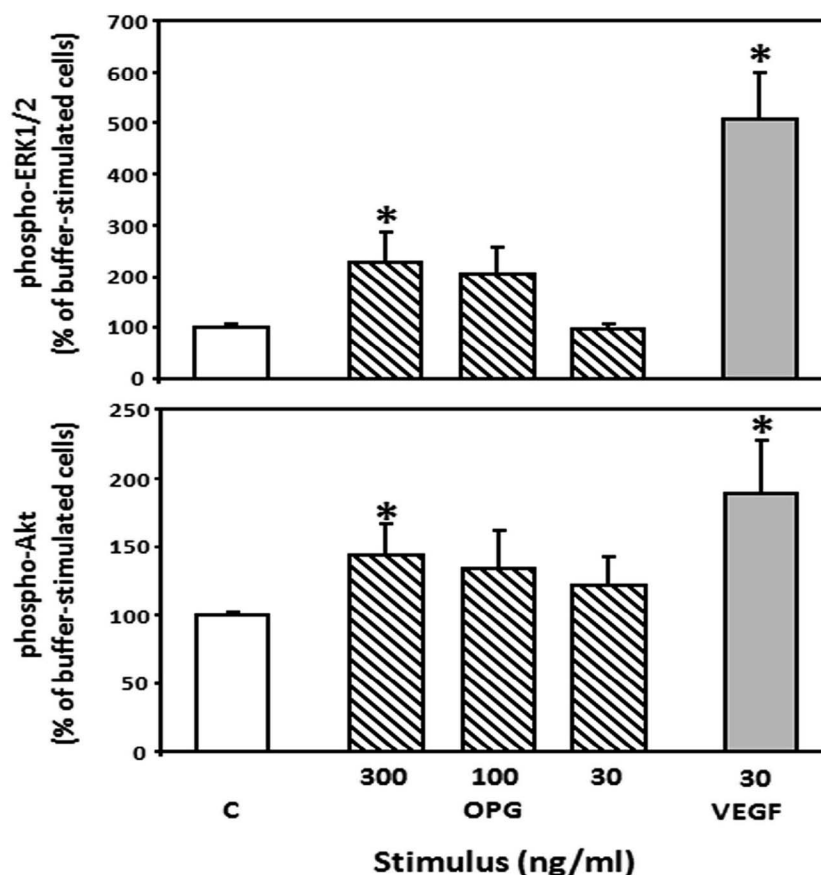


FIGURE 10. Osteoprotegerin activates the intracellular kinases Akt and ERK1/2 in HRMEC. HRMEC were stimulated with OPG (at concentrations of 300, 100, or 30 ng/mL) or with VEGF (30 ng/mL) for 15 minutes. The phospho-ERK1/2-to-total protein content ratio (*upper panel*) or phospho-Akt-to-total protein content ratio (*lower panel*) was calculated for cell lysates of HRMEC after 15 minutes of incubation with the applied stimulus. Results represent a percentage (mean \pm SEM) of the kinase phosphorylation status after buffer (C) treatment. $n = 4$; * $P < 0.05$.

fold higher than that of VEGF and OPG was not as efficacious as VEGF.

OPG Induces In Vivo Angiogenesis

Finally, we evaluated the angiogenic activity of OPG in protein gelatin plugs implanted subcutaneously in mice. In addition to control plugs (no extra stimulus; $n = 12$), plugs containing 500 ng/mL OPG ($n = 9$), 250 ng/mL VEGF ($n = 10$), or OPG plus VEGF ($n = 12$) were created. VEGF and the combination of VEGF plus OPG significantly increased the total number of cells recruited to the plugs (Fig. 11). Flow cytometric analysis demonstrated that an important part of the cells present in the plugs expressed CD34 in the absence of CD45, confirming that blood vessels (lined with endothelial cells) developed in the plugs, as could be observed macroscopically. In addition, OPG was able to enhance the effect of VEGF, suggesting that in eyes of diabetic patients where both OPG and VEGF are present, those endothelial growth factors cooperate in the induction of pathological neovascularization.

DISCUSSION

In the present study, we showed that OPG was significantly upregulated in the vitreous fluid from patients with PDR and in the retinas from subjects with diabetes mellitus. As yet it is unclear why the statistical differences observed in the levels of OPG in patients were not observed in the animal model. One possible explanation is the fact that the rat model represents

short-term effects of an acute diabetogenic event, whereas in the patients, the disease evolved over much longer time intervals, eventually years. Using immunohistochemistry, we demonstrated that OPG protein was specifically produced by vascular endothelial cells, myofibroblasts and leukocytes in PDR fibrovascular epiretinal membranes. Our data are in line with those of others who have shown that OPG is expressed by endothelial cells, macrophages and fibroblasts in the microenvironment of tumors and inflammatory disorders.²⁰⁻²⁴

Although OPG was first described as a modulator of bone metabolism, an increasing number of reports now consider OPG also has a function in other biological systems, including promotion of angiogenesis and vasculogenesis.^{13,20} The angiogenic process requires activation and invasion of endothelial cells through their basement membrane, proliferation, migration and re-establishment of cell-cell contacts to form patent tubes. It has been demonstrated that OPG contributes to each of these events. Involvement of the heparin-binding domain of OPG in the proangiogenic activity of OPG was suggested.^{20,25-27} In addition, it was demonstrated that OPG enhanced the proangiogenic effect of VEGF.²⁶ Similarly, in the present study, we demonstrate that OPG potentiated VEGF-induced angiogenesis in protein gelatin plugs implanted subcutaneously in mice. Additionally, OPG protects endothelial cells from apoptosis induced by growth factor withdrawal.²⁸ OPG's endothelial survival function appears to derive from its ability to inhibit TNF-related apoptosis inducing ligand (TRAIL)-mediated apoptosis.²⁹ Furthermore, OPG stimulates endothelial colony-forming cell survival, migration, chemotaxis and

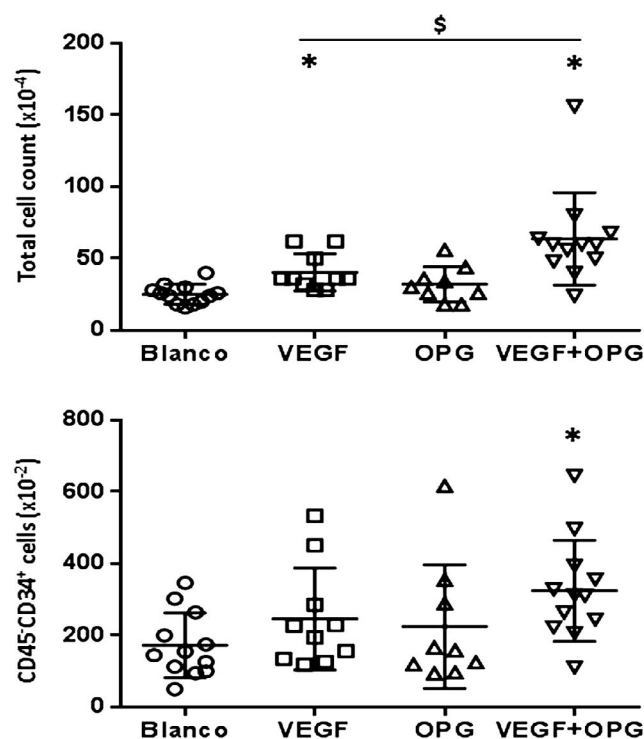


FIGURE 11. Osteoprotegerin enhances the angiogenic activity of VEGF in vivo. A mixture of growth factor-reduced protein gelatin and buffer (blanco), OPG (500 ng/mL), VEGF (250 ng/mL), or OPG plus VEGF respectively, was injected subcutaneously in mice. After 7 days, plugs were resected, and single-cell suspensions were prepared to determine the total number of cells (*upper panel*) or the number of CD45⁺CD34⁺ endothelial cells (*lower panel*) present in the plugs. Each symbol represents an individual plug ($n = 9$ to 12/group), horizontal lines indicate the mean \pm SD. Statistically significant differences are indicated from *control or from \$VEGF; $P < 0.05$.

vascular cord formation suggesting that OPG is involved in vasculogenesis.^{30,31}

In the present study, we found a significant positive correlation between the vitreous fluid levels of OPG and those of the angiogenic and inflammatory biomarkers VEGF and MCP-1/CCL2. Using immunohistochemistry, we demonstrated a significant positive correlation between the level of vascularization in PDR epiretinal membranes and the number of blood vessels and stromal cells expressing OPG. Taken together, these findings suggest that upregulation of OPG in the ocular microenvironment of PDR patients might contribute to the progression of angiogenesis associated with PDR. To corroborate the findings at the cellular level, stimulation with OPG induced HRMEC migration, a key early step in angiogenesis. In addition, we showed that OPG can activate proliferative (ERK1/2) and survival (Akt) signaling pathways in HRMEC, further underscoring its role as a promoter of PDR angiogenesis. Our data are consistent with previous reports that demonstrated the ability of OPG to induce activation of the angiogenic signaling pathways ERK1/2 and Akt in endothelial cells.^{27,31,32}

Chronic low-grade subclinical inflammation plays a central role in the development of diabetic retinopathy.³³ In this respect, it should be emphasized that OPG release by endothelial cells is significantly upregulated by the proinflammatory cytokines TNF- α , IL-1 α , and IL-1 β ^{18,26,34} as also shown in this study for HRMEC. These findings suggest that inflammation might be involved in diabetes-induced retinal endothelial structural damage by inducing OPG expression. Elevated levels of OPG have been observed in chronic inflammatory diseases,^{35–38} suggesting that OPG is a marker of inflammation. Previous reports demonstrated that OPG has proinflammatory properties by activation of classic nuclear

factor-kappa B (NF- κ B) signaling.³⁹ Furthermore, a pathologic role of OPG was also supported by an in vitro study showing that administration of OPG promoted the adhesion of leukocytes to endothelial cells. There is evidence that the heparin-binding domain of OPG is involved in mediating its pro-adhesive activity.⁴⁰ OPG also stimulates upregulation of the endothelial adhesion molecules intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin in TNF- α -activated endothelial cells and facilitates binding of monocytes.⁴¹ Because an abnormal increase of leukocyte/endothelial cell adhesion is considered an early step in diabetes-induced retinal vasculopathy,³³ these data suggest that pathological increase of OPG in the ocular microenvironment of patients with PDR may contribute to the inflammatory status of the endothelium in PDR.

In conclusion, our findings suggest that OPG may be specifically involved in promoting the progression of pathologic neovascularization in the ocular microenvironment of PDR patients. Therapeutic strategies aimed to decrease OPG levels may be suitable for improving the vascular function in diabetic retina.

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References

1. Abu El-Asrar AM, Struyf S, Verbeke H, Van Damme J, Geboes K. Circulating bone-marrow-derived endothelial precursor cells contribute to neovascularization in diabetic epiretinal membranes. *Acta Ophthalmol*. 2011;89:222-228.
2. Abu El-Asrar AM, Struyf S, Opdenakker G, Van Damme J, Geboes K. Expression of stem cell factor/c-kit signaling pathway components in diabetic fibrovascular epiretinal membranes. *Mol Vis*. 2010;16:1098-1107.
3. Abu El-Asrar AM, De Hertogh G, van den Eynde K, et al. Myofibroblasts in proliferative diabetic retinopathy can originate from infiltrating fibrocytes and through endothelial-to-mesenchymal transition (EndoMT). *Exp Eye Res*. 2015;132:179-189.
4. Simó R, Carrasco E, García-Ramírez M, Hernández C. Angiogenic and antiangiogenic factors in proliferative diabetic retinopathy. *Curr Diabetes Rev*. 2006;2:71-98.
5. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996;86:353-364.
6. Spranger J, Pfeiffer AF. New concepts in pathogenesis and treatment of diabetic retinopathy. *Exp Clin Endocrinol Diabetes*. 2001;109(suppl 2):S438-S450.
7. Abu El-Asrar AM, Nawaz MI, De Hertogh G, et al. S100A4 is upregulated in proliferative diabetic retinopathy and correlates with markers of angiogenesis and fibrogenesis. *Mol Vis*. 2014;20:1209-1224.
8. Abu El-Asrar AM, Nawaz MI, Kangave D, Siddiquei MM, Geboes K. Angiogenic and vasculogenic factors in the vitreous from patients with proliferative diabetic retinopathy. *J Diabetes Res*. 2013;2013:539658.
9. El-Asrar AM, Nawaz MI, Kangave D, et al. High-mobility group box-1 and biomarkers of inflammation in the vitreous from patients with proliferative diabetic retinopathy. *Mol Vis*. 2011;17:1829-1838.
10. Nawaz MI, Van Raemdonck K, Mohammad G, et al. Autocrine CCL2, CXCL4, CXCL9 and CXCL10 signal in retinal endothelial cells and are enhanced in diabetic retinopathy. *Exp Eye Res*. 2013;109:67-76.
11. Abu El-Asrar AM, Mohammad G, Nawaz MI, et al. Relationship between vitreous levels of matrix metalloproteinases and vascular endothelial growth factor in proliferative diabetic retinopathy. *PLoS One*. 2013;8:e85857.
12. Abu El-Asrar AM, Alam K, Nawaz MI, et al. Upregulated expression of heparanase in the vitreous of patients with proliferative diabetic retinopathy originates from activated endothelial cells and leukocytes. *Invest Ophthalmol Vis Sci*. 2015;56:8239-8247.
13. Reid P, Holen I. Pathophysiological roles of osteoprotegerin (OPG). *Eur J Cell Biol*. 2009;88:1-17.
14. Zauli G, Melloni E, Capitani S, Secchiero P. Role of full-length osteoprotegerin in tumor cell biology. *Cell Mol Life Sci*. 2009;66:841-851.
15. Nelson CA, Warren JT, Wang MW, Teitelbaum SL, Fremont DH. RANKL employs distinct binding modes to engage RANK and the osteoprotegerin decoy receptor. *Structure*. 2012;20:1971-1982.
16. Vitovski S, Phillips JS, Sayers J, Croucher PI. Investigating the interaction between osteoprotegerin and receptor activator of NF- κ B or tumor necrosis factor-related apoptosis-inducing ligand: evidence for a pivotal role for osteoprotegerin in regulating two distinct pathways. *J Biol Chem*. 2007;282:31601-31609.
17. Blázquez-Medela AM, López-Novoa JM, Martínez-Salgado C. Osteoprotegerin and diabetes-associated pathologies. *Curr Mol Med*. 2011;11:401-416.
18. Secchiero P, Corallini F, Pandolfi A, et al. An increased osteoprotegerin serum release characterizes the early onset of diabetes mellitus and may contribute to endothelial cell dysfunction. *Am J Pathol*. 2006;169:2236-2244.
19. Carrasco E, Hernández C, de Torres I, Farrés J, Simó R. Lowered cortistatin expression is an early event in the human diabetic retina and is associated with apoptosis and glial activation. *Mol Vis*. 2008;14:1496-1502.
20. Cross SS, Yang Z, Brown NJ, et al. Osteoprotegerin (OPG): a potential new role in the regulation of endothelial cell phenotype and tumour angiogenesis? *Int J Cancer*. 2006;118:1901-1908.
21. Haynes DR, Barg E, Crotti TN, et al. Osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathies and osteoarthritis and normal controls. *Rheumatology*. 2003;42:123-134.
22. Tunyogi-Csapo M, Kis-Toth K, Radacs M, et al. Cytokine-controlled RANKL and osteoprotegerin expression by human and mouse synovial fibroblasts. *Arthritis Rheum*. 2008;58:2397-2408.
23. Wada N, Maeda H, Yoshimine Y, Akamine A. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF- κ B ligand in periodontal ligament fibroblasts through the induction of interleukin-1 beta and tumor necrosis factor- α . *Bone*. 2004;35:629-635.
24. Simionescu A, Simionescu DT, Vyavahare NR. Osteogenic responses in fibroblasts activated by elastin degradation products and transforming growth factor- β 1: role of myofibroblasts in vascular calcification. *Am J Pathol*. 2007;171:116-123.
25. Goswami S, Sharma-Walia N. Osteoprotegerin secreted by inflammatory and invasive breast cancer cells induces aneuploidy, cell proliferation and angiogenesis. *BMC Cancer*. 2015;15:935.
26. Reid PE, Brown NJ, Holen I. Breast cancer cells stimulate osteoprotegerin (OPG) production by endothelial cells through direct cell contact. *Mol Cancer*. 2009;8:49.
27. Kobayashi-Sakamoto M, Isogai E, Holen I. Osteoprotegerin induces cytoskeletal reorganization and activates FAK, Src, and ERK signaling in endothelial cells. *Eur J Haematol*. 2010;85:26-35.
28. Malyankar UM, Scatena M, Suchland KL, Yun TJ, Clark EA, Giachelli CM. Osteoprotegerin is an $\alpha_v\beta_3$ -induced, NF- κ B-dependent survival factor for endothelial cells. *J Biol Chem*. 2000;275:20959-20962.
29. Pritzker LB, Scatena M, Giachelli CM. The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. *Mol Biol Cell*. 2004;15:2834-2841.
30. Benslimane-Ahmim Z, Heymann D, Dizier B, et al. Osteoprotegerin, a new actor in vasculogenesis, stimulates endothelial colony-forming cells properties. *J Thromb Haemos*. 2011;9:834-843.
31. Benslimane-Ahmim Z, Poirier F, Delomenie C, et al. Mechanistic study of the proangiogenic effect of osteoprotegerin. *Angiogenesis*. 2013;16:575-593.
32. McGonigle JS, Giachelli CM, Scatena M. Osteoprotegerin and RANKL differentially regulate angiogenesis and endothelial cell function. *Angiogenesis*. 2009;12:35-46.

33. Joussen AM, Poulaki V, Le ML, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J*. 2004;18:1450-1452.
34. Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P. Receptor activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. *J Biol Chem*. 2001;276:20659-20672.
35. Asanuma Y, Chung CP, Oeser A, et al. Serum osteoprotegerin is increased and independently associated with coronary-artery atherosclerosis in patients with rheumatoid arthritis. *Atherosclerosis* 2007;195:e135-e141.
36. Ziolkowska M, Kurowska M, Radzikowska A, et al. High levels of osteoprotegerin and soluble receptor activator of nuclear factor kappa B ligand in serum of rheumatoid arthritis patients and their normalization after anti-tumor necrosis factor alpha treatment. *Arthritis Rheum*. 2002;46:1744-1753.
37. Kwok SK, Shin YJ, Kim HJ, et al. Circulating osteoprotegerin levels are elevated and correlated with antiphospholipid antibodies in patients with systemic lupus erythematosus. *Lupus*. 2009;18:133-138.
38. Golledge J, McCann M, Mangan S, Lam A, Karan M. Osteoprotegerin and osteopontin are expressed at high concentrations within symptomatic carotid atherosclerosis. *Stroke*. 2004;35:1636-1641.
39. Nahidi L, Leach ST, Lemberg DA, Day AS. Osteoprotegerin exerts its pro-inflammatory effects through nuclear factor-kB activation. *Dig Dis Sci*. 2013;58:3144-3155.
40. Zauli G, Corallini F, Bossi F, et al. Osteoprotegerin increases leukocyte adhesion to endothelial cells both in vitro and in vivo. *Blood*. 2007;110:536-543.
41. Mangan SH, Van Campenhout A, Rush C, Golledge J. Osteoprotegerin upregulates endothelial cell adhesion molecule response to tumor necrosis factor-alpha associated with induction of angiopoietin-2. *Cardiovasc Res*. 2007;76:494-505.